LIQUID CHROMATOGRAPHIC ENANTIOSEPARATION OF ACTIVE PHARMACEUTICAL INGREDIENTS AND STERICALLY HINDERED AMINO ACIDS

Ph.D. Thesis

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Introduction and purposes

Physical, biological and chemical properties determined by the symmetry and asymmetry play an especially important role in nature. The natural compounds are mainly chiral so it is easy to understand, that uptake of the enantiomers of a chiral compound in the organism can cause different effects, they can evolve different interactions and also the route of the metabolism of the stereoisomers can be different. Accordingly, a demand arose in the pharmaceutical and other therapeutic fundamental research for the preparation of the stereoisomers in their enantiomerically pure form, and also for the analytical quantification and qualification of the pure chiral product.

In the peptide-based receptor-research the receptors interact with peptides of known conformation. The way for the preparation of peptides with known conformation is the application of sterically hindered amino acids. These amino acids have rigid structures and have special functional groups that can form conformational blocks in the structure of peptides. The preparation of peptides with known structures requires application of enantiomerically pure amino acids.

There are several possibilities to prepare pure enantiomers, for example using enantioselective synthetic routes (application of stereoselective catalysts) or separation of enantiomers after preparation of racemic mixtures. The separations can be carried out by the traditional resolvation processes or using preparative chiral chromatographic techniques.

The chromatographic processes exhibited important role not only in the preparation of the enantiomers, but in the quality control of the chiral products. Nowadays the chiral high-performance liquid chromatography

(HPLC) is one of the most dynamically developing and the most widely applied branch of the chiral analytics.

The aim of our work was to develop HPLC methods for the enantioseparation of a chiral antitumor active pharmaceutical ingredient (API), its starting materials and side products. Moreover our purposes included the HPLC enantioseparation of several unnatural sterically hindered amino acids. Among these amino acids were some spin-labeled β-amino acids and some specially substituted glycine (Gly) and alanine (Ala) analogues. Our additional aim was to investigate the chromatographic properties and retention mechanism of a quinine-based anion-exchanger type chiral stationary phase (CSP).

Experimental

Direct and indirect chromatographic methods were applied during the investigations.

Three different type of chiral derivatizing agents (CDAs) were used in case of the indirect chromatographic methods, the active fluorine containing 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, i.e. Marfey's reagent (FDAA), the isothiocyanate-group containing 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and the active ester containing (*S*)-*N*-(4-nitrophenoxycarbonyl)-phenylalanine methoxyethyl ester (NIFE).

For direct chromatographic methods different types of CSPs were applied, including cellulose-based, α- and β-cyclodextrin-based, macrocyclic glycopeptide-based and quinine-based stationary phases. All of the chiral selectors were covalently bonded to the silica support.

The applied apparatus were as follows:

I. Chromatographic system: M-600 low pressure quaternary gradient pump, M-996 photodiode array detector, Millenium 32 Chromatography Manager software (Waters, Milford, USA).

II: Chromatographic system: 1525 high pressure binary gradient pump, 487 dual wavelength detector, 717 autosampler, in-line degasser, Breeze Chromatography Manager software (Waters, Milford, USA).

Both systems included a 7125 manual injector with 20 μl loop (Rheodyne, Cotati, USA).

MK-70 cooling-heating thermostat (Mechanik Prüfgeräte, Medlingen, Germany).

420 A type precision pH-meter (Orion, Beverly, USA).

Results

Bicalutamide is a nonsteroidal antiandrogen active pharmaceutical ingredient and it is successfully applied in the therapy of prostate cancer. Our task was to develop chiral HPLC procedures in order to control the enantiopurity of the starting materials, the intermediates and the side products of the synthesis of bicalutamide.

1. The enantioseparation of bicalutamide and one of its intermediates and a side product was carried out on a Chiralcel™ OD-RH column. The optimized chromatographic parameters were described.

2. The usefulness of various macrocyclic glycopeptide-based stationary phases during enantioseparation of bicalutamide and of its starting materials, intermediates and side products was investigated. Among the applied columns the Chirobiotic™ T and Chirobiotic™ TAG exhibited the best efficiency. The Chirobiotic™ T demonstrated outstanding selectivity during normal-phase analyses. In the reversed-phase investigations the Chirobiotic™ TAG showed similarly excellent enantioselectivity.

3. The enantioseparation of reactive, therefore hardly analyzable intermediates was carried out on a β-cyclodextrin-based Cyclobond™ I 2000 SN column.

4. We attempted to accomplish the enantioseparation of the stereoisomers of the five investigated compounds in one chromatographic run, but it failed due to solvation problems. At least two injections were needed to separate the stereoisomers of all compounds.

5. The elution sequence of the enantiomers of all compounds on all of the investigated stationary phases were determined. The chiral contaminations in the enantiopure samples were found to be less than 1%.

Stable nitroxide free radicals are applied as spin traps of other radicals in biological studies. The optically active ones are used as chiral oxidizing agents and chiral derivatizing agents of achiral radicals. Some types of spin-labeled amino acids are often built in peptides for biological and conformational EPR studies.

6. The direct chiral separation of Fmoc-protected spin-labeled β-amino acids was carried out on a Chiralcel™ OD-RH column in reversed-phase mode. The separation was optimized by varying the mobile phase composition and column temperature.

7. Indirect chiral chromatographic processes were developed for the separation of stereoisomers of unprotected spin-labeled β-amino acids. The effectiveness of three CDAs – FDAA, GITC and (*S*)-NIFE – was tested. The influence of the changing reaction conditions for the conversion of diastereomeric derivatives and for the racemization of amino acids was investigated.

7.1. The FDAA exhibited the lowest applicability for the derivatization and enantioseparation of spin-labeled β-amino acids, because of its mild reactivity.

7.2. When using GITC, acceptable yield of conversion but high degree of racemization was observed. To avoid racemization, milder reaction conditions were applied and the yield of conversion also decreased.

7.3. Among the investigated CDAs the (*S*)-NIFE was the only one, which exhibited excellent reactivity and selectivity for the separation of enantiomers of cyclic, spin-labeled β-amino acids.

8. The elution sequences of the stereoisomers were determined for each developed method.

9. The enantiopurity was determined for both the direct and indirect methods and comparable results were obtained.

The enantioseparation of secondary α-amino acids ("imino acids") were accomplished on two, structurally similar macrocyclic glycopeptide-based stationary phases.

10. Direct chromatographic processes were developed for the separation of enantiomers of these type of amino acids.

11. The influence of the mobile phase composition and the pH of the mobile phase on the chromatographic parameters were investigated. The change of pH revealed that electrostatic interactions forming, via the carboxylic group of the stationary phase, exhibited an important role in the retention process.

12. The separation ability of the two glycopeptide-based stationary phases was compared for the separation of enantiomers of imino acids. Our observations were similar to those described in the literature for the β-amino acids. Accordingly, the native teicoplanin selector was often proved higher selectivity than the teicoplanin aglycon. This behavior can be explained by the hard fitting of the rigid and sterically hindered β-amino- and imino acids in the hydrophobic cavities of the CSPs. In the case of teicoplanin, despite the covered hydrophobic cavities (by sugar moieties), the CSP exhibited

higher efficiency, probably due to hydrogen bond formation or increased steric interactions.

Four kinds of acylated derivatives of sixteen glycine and phenylalanine analogue amino acids were separated. In our work on a quinine-carbamate based CSP, the influence of the column temperature, the mobile phase composition and the structure of the sample molecules on chiral recognition process were investigated.

13. For these purposes four kinds of acylated derivatives [benzyloxy-carbonyl (Z), the 3,5-dinitro-benzyloxy-carbonyl (DNZ), the benzoyl (Bz) and the 3,5-dinitro-benzoyl (DNB)] of the investigated amino acids were prepared. Our general observation was that the retention of enantiomers increased in the sequence Z < Bz < DNZ < DNB.

14. The chromatograms of the investigated compounds were recorded at eight temperatures in the range of 278-343 K. With increasing temperature the values of the retention factors (k') and selectivity factors (α) were decreased, however the resolution (R_S) showed a maximum curve.

15. In order to investigate the role of the hydrophobic interactions the 10-based logarithm of distribution-ratio (log P) of the investigated amino acids in 1-octanol/water mixture (log P) were calculated. The calculated log P values were compared with retention factors for Z and DNZ derivatives of several compounds in different mobile phase systems. Our conclusion was that hydrophobic interactions play important role in the retention process, but they have small selectivity. Other interactions should be considered, which have larger selectivity, especially electrostatic, π-acidic π-basic interactions and hydrogen bond interactions.

16. The enthalpy (ΔH^o) and entropy change (ΔS^o) as well as the difference of change in enthalpy [$\Delta(\Delta H^o)$], entropy [$\Delta(\Delta S^o)$] and Gibbs free

energy [$\Delta(\Delta G^o)$] with respect to each pair of enantiomers were calculated from chromatographic parameters measured at different temperatures.

16.1. The π-acidic π-basic interactions existing between the π-basic quinoline ring of the selector and the π-acidic nitro-substituted aromatic rings of DNZ and DNB derivatized analytes greatly contributeed to the increase of both the retention and the selectivity.

16.2. The higher rigidity of molecular structure generally accounts for the chiral recognition but the influence of steric interactions can be favorable and unfavorable, too.

16.3. The formation of hydrogen bonds generally contributes to the increase of retention but it has less importance in the chiral recognition.

List of publications

Publications based on the Ph.D. work:

1. K. Wright, M. Crisma, C. Toniolo, **R. Török**, A. Péter, M. Wakselman, J.-P. Mazaleyrat
4-Amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (β-TOAC), the first spin-labelled, cyclic, chiral β-amino acid resolved in an enantiomerically pure state
Tetrahedron Letters, **44**, 3381-3384, 2003 IF: 2,326

2. K. Wright, F. Formaggio, C. Toniolo, **R. Török**, A. Péter, M. Wakselman, J.-P. Mazaleyrat
First access to the spin-labelled β-amino acid POAC in an enantiopure state by resolution through its binaphthyl esters
Tetrahedron Letters, **44**, 4183-4186, 2003 IF: 2,326

3. A. Péter, **R. Török**, K. Wright, M. Wakselman, J.-P. Mazaleyrat
Liquid chromatographic enantioseparation of spin-labelled β-amino acids
Journal of Chromatography A, **1021**, 1-10, 2003 IF: 2,922

4. A. Péter, **R. Török**, D. W. Armstrong
Direct high-performance liquid chromatographic separation of unusual secondary amino acids and a comparison of the performances of Chirobiotic T and TAG columns
Journal of Chromatography A, **1057**, 229-235, 2004 IF: 3,359

5. **R. Török**, Á. Bor, Gy. Orosz, F. Lukács, D. W. Armstrong, A. Péter
High-performance liquid chromatographic enantioseparation of bicalutamide and its related compounds
Journal of Chromatography A, **1098**, 75-81, 2005 IF: 3,096

6. **R. Török**, R. Berkecz, A. Péter
High-performance liquid chromatographic enantioseparation of α-substituted glycine analogs on a quinine-based anion-exchanger chiral stationary phase under variable temperature conditions
Journal of Chromatograhy A, **1120**, 61-68, 2006 IF: 3,096

7. **R. Török**, R. Berkecz, A. Péter
Enantioseparation of phenylalanine analogs on a quinine-based anion-exchanger chiral stationary phase. Structure and temperature effects
Journal of Separation Science, Accepted for publication IF: 1,829

Sum of impact factors: 17,125

Other publications:

1. R. Berkecz, **R. Török,** I. Ilisz, E. Forró, F. Fülöp, D. W. Armstrong, A. Péter
High-performance liquid chromatographic enantioseparation of β-lactam and β-amino acid stereoisomers and a comparison of the performances of macrocyclic glycopeptide-based columns
Chromatographia, **63**, S37-S43, 2006 IF: 0,959

Conference presentations and posters:

1. **R. Török**, A. Péter
   Develoment of new liquid chromatographic processes for the enantioseparation of spin-labeled β-amino acids
   XXVI. Kémiai Előadói Napok (KEN)
   Szeged (27-29 October 2003) Hungary Presentation

2. A. Felinger, E. Vékes, **R. Török**, A. Péter
   Temperature effects on the heterogenous adsorption in chiral separation
   HPLC-2004: 28th International Symposium and Exhibition on High performance Liquid Phase Separation and Related Techniques
   Philadelphia (12-18 June 2004) USA Poster

3. K. Wright, A. de la Croix de Castries, M. Sarciaux, M. Vakselman, J.-P. Mazaleyrat, **R. Török**, A. Péter, M. Crisma, F. Formaggio, C. Toniolo
   Enantiomerically pure, cyclic, spin-labelled β-amino acids; trans-POAC and cis/trans-β-TOAC
   3rd International and 28th European Peptide Symposium
   Prague (5-10 September 2004) Czech Republic Poster

4. **R. Török**, N. Maier, W. Lindner, A. Péter
   Effects of molecular structure and temperature on retention of acylated glycine and alanine analogs on a quinine-based anion-exchanger chiral stationary phase
   HPLC-2005: 29th International Symposium on High Performance Liquid Phase Separations and Related Techniques
   Stokholm (26-30 June 2005) Sweden Poster

5. **R. Török**, R. Berkecz, I. Ilisz, E. Forró, F. Fülöp, D. W. Armstrong, A. Péter
   High-performance liquid chromatographic enantioseparation of β-lactam and β-amino acid stereoisomers and a comparison of the performances of macrocyclic glycopeptide-based columns
   6th Balaton Symposium on High-Performance Separation Methods
   Siófok (7-9 September 2005) Hungary Poster

6. A. Péter, **R. Török**, Á. Bor, G. Orosz, F. Lukács, D. W. Armstrong
   High-performance liquid chromatographic enantioseparation of bicalutamide and its related compounds
   17th International Symposium on Chiral Discrimination
   Parma (11-14 September 2005) Italy Poster

7. **R. Török**, A. Péter
Chiral liquid chromatographic separations on macrocyclic antibiotic-based stationary phases
VI. Elválasztástudományi Ankét
Budapest (23 February 2006) Hungary Presentation